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of

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for

SODIUM ACTIVATION OF AMYLASE

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BACKGROUND OF THE INVENTION

1. The Field of the Invention

The present invention relates to methods, compositions, and assays for the determination of chemical components in biological samples. More particularly, the present invention relates to colorimetric assays based on the sodium activation of amylase.

2. The Relevant Technology

Chloride ion is the major extracellular negative ion in the human body. Its main function is to maintain electrical neutrality by acting as a counter-ion to sodium. Accordingly, chloride ion levels often accompany sodium losses and excesses. Chloride ion also helps regulate acid-base balances by entering cells in response to rising carbon dioxide levels. As carbon dioxide increases, bicarbonate moves from the intracellular space to the extracellular space. In response, chloride tends to enter the cells.

Therefore, there are various circumstances where it is important to analyze serum and other bodily fluids to determine the amount of chloride ion. For example, hyperchloremia (high serum chloride) may indicate chronic hyperventilation, Cushing's syndrome, dehydration, eclampsia, excess infusion of normal saline, kidney dysfunction, metabolic acidosis, or renal tubular acidosis. Hypochloremia (low serum chloride) may indicate Addison's disease, burns, chronic respiratory acidosis (chronic hypoventilation), congestive heart failure, excessive sweating, gastric suction, over hydration, salt-losing nephritis, syndrome of inappropriate ADH secretion, or vomiting.

In response to this need, there have been developed various methods and devices to analyze bodily fluids and determine the amount of chloride ion in a sample. One early method was a colorimetric test of free chlorine. It detected the presence of the chloride ion

1 by using a soluble silver salt and tolidine. However, this method lacked the precision
2 required by most applications.

3 The need for more precise chloride ion measurements has led to the development of
4 more accurate tests through a variety of methods. Most current methods for the
5 determination of chloride ion are based on either electric or chemical methods. Electric
6 methods include coulometric titration and the ion selective electrode method. The
7 coulometric titration method is considered the most reliable method, if not the quickest. It
8 can be performed with either manual or semi-automatic methods, but is difficult to perform
9 in an automatic analytical system. The ion selective electrode method can have specificity
10 problems and is prone to interference from proteins and surfactants.

11 The principal chemical method is colorimetry, wherein the concentration of the
12 chloride ion is measured according to changes in color density. The colorimetry method is
13 considered the most effective test for simple applications because it is less complicated than
14 coulometric or ion selective electrode methods. The most common chloride determination
15 colorimetry methods react mercuric thiocyanate and chloride ion to produce thiocyanate ion,
16 which then forms a complex with ferric ion. The result is a characteristic red orange color,
17 which deepens as the concentration of the chloride ion becomes higher, thus enabling
18 colorimetry. Nevertheless, this method has drawbacks because both mercuric ions and the
19 thiocyanate ions are harmful to the environment. As a result, each use of the reagents
20 creates additional waste that requires costly care and treatment. Therefore, there is a need
21 for colorimetric methods to determine chloride ion concentration that avoid the mercuric and
22 thiocyanate ions.

23 U.S. Patent No. 5,229,270 to Ono et al. (hereinafter "Ono") discloses an
24 environmentally safe quantitative assay and reagent for the determination of chloride ion in

1 bodily fluids. *Ono* uses a reagent which contains deactivated α -amylase, a compound
2 capable of chelating calcium ion, a calcium chelate ion, a calcium chelate compound, and an
3 α -amylase measuring substance. The method comprises the steps of: (a) contacting a bodily
4 fluid sample suspected of containing chloride ions with a reagent which comprises a
5 compound capable of forming a chelate with a calcium ion, deactivated α -amylase, a
6 calcium chelate compound, and an α -amylase activity-measuring substance; (b) determining
7 the quantity of α -amylase activity formed due to the presence of chloride ions in the bodily
8 fluid sample, which is directly proportional to the amount of chloride ions present in the
9 bodily fluid sample; and (c) determining the quantity of the chloride ions from the quantity
10 of the α -amylase activity by referring to a calibration curve. This method is capable of
11 automation and has high ion specificity.

12 It has previously been shown that α -amylase contains one chloride ion binding site
13 per molecule. One early approach at describing the chloride ion effects on α -amylase is
14 described in Lifshitz, Ruth, Levitski, Alexander, *Identity and Properties of the Chloride*
15 *Effector Binding Site in Hog Pancreatic α -Amylase*, Biochemistry, Vol. 15, No. 9, 1976.
16 (hereinafter, "*Lifshitz*"). *Lifshitz* is specifically directed to determine the chloride ion
17 binding site in α -amylase. In reaching their conclusions, *Lifshitz* discusses various
18 compounds and their effects upon the chloride ion binding site. For example, *Lifshitz*
19 teaches that calcium-free α -amylase is unable to bind chloride ion. Similarly, *Lifshitz* tested
20 sodium fluoride (fluoride ion) and sodium acetate (acetate ion) to determine their effect
21 upon the chloride ion affinity of deactivated α -amylase. *Lifshitz* determined that neither
22 fluoride ion nor sodium acetate had any appreciable effect upon the chloride ion affinity of
23 deactivated α -amylase. *Lifshitz* at 1990.

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1 Notwithstanding the prior methods for assaying chloride ions using calcium-
2 activated α -amylase, there remains a continuing need for alternative systems for assaying
3 chloride.

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1 concentration of sodium ions in bodily fluid samples. The method comprises first preparing
2 an enzyme reagent which includes α -amylase that is substantially calcium-free and an α -
3 amylase activity detecting substrate. Next, the enzyme reagent, chloride ion, and a sample
4 containing sodium ion to be assayed are combined, wherein the chloride ion is present in a
5 higher concentration than the sodium ion. The quantity of α -amylase formed due to the
6 presence of sodium ions and chloride ions in the sample is then assayed. Finally, the
7 quantity of the sodium ions is determined by reference to the assay of α -amylase.

8 These and other objects and features of the present invention will become more fully
9 apparent from the following description and appended claims, or may be learned by the
10 practice of the invention as set forth hereinafter.

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DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to methods, systems, and compositions based on the discovery that sodium ion functions as an activator for α -amylase. More particularly, the present invention relates to colorimetric assays based on the discovery that sodium activates amylase even in the absence of calcium. Preferred aspects of the invention are directed to the determination of chloride ion and sodium ion in biological samples. In particular, assays where calcium activation of amylase is undesirable can utilize the sodium activation of amylase as a substitute.

With regards to the determination of chloride ion in a bodily fluid sample, a calcium binding compound is first used to remove all calcium from the bodily fluid sample so as to ensure the enzyme is not activated by the presence of calcium in the system. The addition of chloride ion and excess sodium ion then enables sodium ion to bind with deactivated α -amylase thus activating the α -amylase at a level proportional to the amount of chloride ion. Although the present invention is not limited to any particular theory, it is believed that the sodium ion activates amylase by a mechanism similar to that of the calcium ion.

The novel methods and systems according to the invention are preferably performed by colorimetry, using measurements of α -amylase activity to indirectly measure the chloride ion concentration. The methods and systems involve one or more measurements of a detectable product of α -amylase activity at a specified time or times after the enzymatic reaction is initiated. From correlations with known data, the system assays for total chloride ion. Alternatively, the system can be used to assay for sodium ion concentration using excess chloride ion in a sample with an unknown sodium ion concentration.

The invention can be used with a variety of samples. Of particular interest are biological samples known to contain chloride ion in measurable quantities. For example,

1 serum, plasma, and urine are known to have quantifiable amounts of chloride that can be
2 used as a sign of metabolic function of electrolytes. Of course, the present invention can be
3 used in other applications where chloride ion determination is desired. Therefore, the
4 invention also encompasses a method for the determination of chloride ion concentration of
5 non-bodily fluids as well.

6 Referring now to the reagents according to the present invention, one method
7 according to the present invention comprises first preparing an enzyme reagent which
8 includes α -amylase that is substantially calcium-free and an α -amylase activity detecting
9 substrate. Next, the enzyme reagent, sodium ion, and a sample containing chloride ion to be
10 assayed are combined, wherein the sodium ion is present in a higher concentration than the
11 chloride ion. The quantity of α -amylase formed due to the presence of sodium ions and
12 chloride ions in the sample is then assayed. Finally, the quantity of the chloride ions is
13 determined by reference to the assay of α -amylase.

14 The sodium ion compound is preferably included either by addition to the enzyme
15 reagent or by later addition to the combined solution.

16 As indicated, the enzyme used according to the present invention is α -amylase.
17 Alpha-amylases are enzymes that catalyze the hydrolysis of complex carbohydrates into
18 maltose and residual glucose. It is well known that α -amylase is active when coupled with
19 the calcium ion and it has been believed that removal of calcium ion results in deactivated α -
20 amylase (hereinafter "apo-AMY"). However, it has been surprisingly discovered that
21 sodium ion also functions as an activator of α -amylase, even in the absence of calcium ions.

22 A preferred class of substances that removes calcium from α -amylase are chelating
23 compounds, such as ethylenediaminetetraacetic acid (EDTA), that are capable of forming a
24 chelate with calcium. Calcium-chelating compounds form a highly stable complex between

1 a calcium ion and more than one organic group to form a ring, thus depriving intermixed
2 chemicals of calcium. In sufficiently high concentrations, chelating compounds
3 substantially deactivate the α -amylase by chelating substantially all of the calcium.
4 However, chelating compounds do not permanently bind the calcium ion. When apo-AMY
5 in the presence of calcium-EDTA, for example, is reacted with high concentration chloride
6 ion, the apo-AMY is again coupled with the calcium ion to form active-AMY. See A.
7 Levitzki and M. L. Steer, *The Allosteric Activation of Mammalian α -Amylase by Chloride*,
8 *Eur. J. Biochem.* 41, p. 171 (1974). In addition to EDTA, other preferred calcium-chelating
9 compounds include trans-1,2-cyclohexanediamine-N,N,N',N'-tetraacetic acid, glycol ether
10 diamine tetraacetic acid, iminotetraacetic acid, and diaminopropanetetraacetic acid

11 Because of the reversibility of this calcium removal, one has to guard against
12 activation of the amylase by calcium because that would interfere with the assay of this
13 invention. One approach is to maintain a sufficiently high calcium-chelating compound
14 concentration to ensure that all substantially all calcium is bound, thus minimizing the
15 reverse reaction.

16 A calcium-chelate compound is in principle a chelate of the above calcium-chelating
17 compound with calcium ion. Examples of calcium-chelate compounds include calcium
18 ethylenediaminetetraacetate (Ca-EDTA), calcium trans-1,2-cyclohexanediamine-N,N,N',N'-
19 tetraacetate, calcium iminotetraacetate, and calcium diaminopropanetetraacetate. As EDTA
20 is preferable, the use of Ca-EDTA is also preferable.

21 Another class of substances that removes calcium from test samples is compounds
22 that form a covalent bond with calcium. This allows the permanent removal of calcium
23 from the test sample, with no risk of a reverse reaction releasing calcium and destroying the
24 accuracy of the assay.

1 Because α -amylase functions in biological systems to split complex carbohydrates
2 and therefore has biological significance apart from the objects of the present invention,
3 various detecting means have been developed and are known in the art. Alpha-amylase
4 detecting means that are suitable for use in the present invention include substrates that are
5 hydrolyzed by α -amylase and release substances which are detectable by colorimetric
6 methods. In the preferred case, active-AMY hydrolyzes 2-chloro-4-nitrophenyl- α -D-
7 maltotrioxide (CNP3) to form 2-chloro-4-nitrophenol, 2-chloro-4-nitrophenyl- α -D-
8 maltoside (CNP2), maltotriose and glucose. The reaction is depicted in the following
9 equation:

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11 Equation 1

12 Active - AMY + CNP3 \rightarrow 2 - chloro - 4 - nitrophenol + CNP2 + maltotriose + glucose
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14 The rate of formation of the 2-chloro-4-nitrophenol can be measured in a spectrophotometer
15 at 405 nm to give a direct measurement of active-AMY in the sample.
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17 Alternatively, the α -amylase detecting substrate may comprise a substrate
18 composition that utilizes a coupling enzyme. For example, preferred compositions include a
19 substance comprised of 4-nitrophenyl- α -D-maltopentaoside as substrate and α -glucosidase
20 as coupled enzyme, a substance comprised of 2-chloro-4-nitrophenyl- β -D-maltopentaoside
21 as substrate and α -glucosidase and β -glucosidase as coupled enzyme, a substance comprised
22 of 4-nitrophenyl- α -D-maltoheptaoside as substrate and α -glucosidase as coupled enzyme,
23 and a substance comprised of 2-chloro-4-nitrophenyl-b-D-maltoheptaoside as substrate and
24 α -glucosidase and β -glucosidase as coupled enzyme. In these embodiments, the α - and β -

1 glucosidases are coupling enzymes that hydrolyze an intermediate product, such as 4-
2 nitrophenyl- α -D-maltose, 4-nitrophenyl- α -D-maltotriose, 2-chloro-4-nitrophenyl- β -D-
3 maltose or 2-chloro-4-nitrophenyl- β -D-maltotriose, to the final product, i.e. 4-nitrophenol or
4 2-chloro-4-nitrophenol.

5 Of course, one skilled in the art will recognize, in light of the disclosure herein, that
6 other currently known or future developed α -amylase detecting substrate or methods can be
7 used in the present invention.

8 Sodium ion compounds according to the invention include any of a variety of
9 substances that release sodium ion when added to the reagents of the present invention. For
10 example, preferred sodium ion compounds include sodium acetate and sodium citrate.

11 Because the methods of the present invention involve enzymatic reactions, the pH
12 value must be maintained within suitable ranges. Preferably, the pH is maintained between
13 7 and 9. Most conventional buffers can be used to maintain the pH in this range. Examples
14 of suitable buffers include triethanolamine (TEA), 3-morpholinopropane sulfonic acid
15 (MOPS), phosphate buffer, tris-HCl, citrate buffer, and tricine.

16 Other components are also included to maintain the solution at a constant state when
17 a bodily fluid sample is added. Preferred components of the substrate reagent typically
18 include one or more of lithium hydroxide (LiOH), octoxynol (commercially available from
19 Rohm and Haas under the name "Triton X"), trehalose, polyethylene glycol 8000 (PEG
20 8000), and bovine serum albumin (BSA).

21 Other preferred components of the enzyme reagent optionally include one or more of
22 LiOH, dextran, trehalose, PEG 8000, and BSA. Of course, one skilled in the art will
23 recognize that a variety of substances can be used to formulate a composition that will
24 maintain a constant state when a bodily fluid sample is added

1 Advantageously, as can be readily observed from the reagent components cited
2 above, the reagents of this invention do not contain environmentally hazardous materials.
3 Therefore, the present invention has the advantage of simpler handling and disposal
4 requirements than many prior art chloride ion determination methods and reagents.

5 Referring now to the methods for chloride ion determination according to the present
6 invention, one preferred method comprises first preparing an enzyme reagent, which
7 includes α -amylase that is substantially calcium free and an α -amylase activity detecting
8 substrate. The α -amylase that is substantially calcium free is provided by use of one of the
9 calcium binding compounds discussed hereinabove. Next, the enzyme reagent, sodium ion,
10 and a sample suspected of containing chloride ion are combined, wherein the sodium ion is
11 present in a higher concentration than the chloride ion. The quantity of activated α -amylase
12 formed due to the presence of chloride ions in the bodily fluid sample is then determined by
13 assay. Finally, the quantity of chloride ions is indirectly determined from the directly
14 measured activity of the α -amylase. The concentration of chloride ion within a range of
15 about 60-140 mM in a sample can be determined with the above method.

16 In the case of the use of a calcium-chelating compound, the calcium-chelating
17 compound functions not only to initially deactivate the α -amylase, but also to stabilize the
18 apo-AMY. It is difficult to remove all calcium ion from the reagent because the calcium-
19 chelate in the reagent contains calcium. Thus, a small portion of the apo-AMY is supplied
20 with calcium ion and is converted into the active-AMY even in the absence of chloride ion.
21 The result of this unintended reaction is that a blank reagent (blank reaction) in the data
22 obtained, causing inaccuracy of the measurement. The calcium-chelating compound
23 effectively inhibits the reverse reaction of the apo-AMY to active-AMY by preventing the
24 above-mentioned undesirable reaction. Thus, to ensure accuracy, any calcium ion that may

1 be present is minimized by using a sufficient concentration of calcium-chelating compound.

2 Any remaining calcium ion is accounted for by running a blank sample.

3 The concentration of each component in the assay solution is controlled so that the
4 concentration of chloride ions in the sample is rate-controlling. Preferably, the substrate and
5 other necessary reactants are maintained in excess so that the only limiting factor is the
6 chloride ion concentration. Further, the process ideally limits the rate of loss of the
7 detectable reactant so that rate of loss is not necessarily included. The reactants other than
8 chloride ion are present in concentrations sufficiently high to not significantly limit the rate
9 of the reaction over the period of the analysis. Thus, measurement of the α -amylase or
10 product concentration at a specified time or times after the reaction is initiated is a measure
11 of the rate of loss of α -amylase or of production of the detectable product, and provides data
12 which are correlated to chloride ion concentration.

13 Thus, according to the invention, a selected volume of a chloride ion standard or
14 biological fluid sample suspected of containing chloride ion is combined with a selected
15 volume of the above reagents and mixed, initiating the reactions. This method can
16 preferably be performed in an automatic analytical system for serum as is common in the
17 art.

18 The reaction progress can preferably be measured by introducing the mixture to a
19 quartz cuvette, and placing the cuvette in a spectrophotometer. At a specified time or times
20 after the initiation of the reaction, the absorbance at about 405 nm is read and recorded.
21 According to the present invention, measurement of the α -amylase detecting substrates can
22 be performed with spectrophotometers as are currently known in the art. Such
23 spectrophotometers are widely available in chemical and clinical laboratories. Further, this
24 method can preferably be performed in an automatic analytical system for serum as is

1 common in the art. Therefore, the present invention has the advantage that the various
2 apparatuses required to practice the invention are widely available.

3 Preparation of a calibration curve for use with the present invention may be
4 performed in the following manner. First, the reagent to be used in later tests is mixed with
5 a series of known samples containing incremental concentrations of chloride ion. The
6 sample chloride concentrations are selected to roughly correspond to the expected chloride
7 ranges to be tested. For example, chloride ion concentration in serum typically falls within a
8 range of about 70-130 mM. Next, the activity of the α -amylase is then measured in each
9 sample test by the procedures taught hereinabove. Finally, the α -amylase is then plotted in a
10 calibration curve as α -amylase activity versus chloride ion concentration. The chloride ion
11 concentration can then be determined in a sample by locating the absorbance from the
12 sample on the calibration curve showing the absorbance of the standard(s) as a function of
13 chloride ion concentration.

14 Alternatively, chloride ion concentration can be calculated by inserting data for the
15 absorbance of the sample, the absorbance of a standard, and the concentration of the sample
16 into an equation, from which the chloride ion concentration can be calculated. One example
17 equation is:

18 Equation 2

19
20
$$\text{Cl}^- \text{ concentration} = K \frac{(\text{absorbance of sample})(\text{concentration of sample})}{(\text{absorbance of standard})}$$

21 wherein K is a constant dependent upon the components of the assay solution, their
22 concentrations, and the volumes of the sample and assay solution.

23 Yet another chloride ion calculation method is performed by measuring the
24 absorbance at two or more predetermined times following the initiation of the reaction. The

1 absorbance readings must be corrected for any background absorbencies or the reaction rate
2 of the reagent. The corrected absorbance readings are then plotted versus time, a connecting
3 line is drawn, and the slope of the line is calculated. The slope of the line directly correlates
4 to the reaction rate, and can be compared to known slopes to find a similarly determined rate
5 for a standard of known chloride ion concentration.

6 The above reagents and methods can be modified, in view of the disclosure herein,
7 for use in the determination of sodium ion. For example, one preferred method comprises
8 first preparing an enzyme reagent, which includes α -amylase that is substantially calcium-
9 free, an α -amylase activity detecting substrate, and chloride ion. Next, the enzyme reagent ,
10 a sample suspected of containing sodium ion, and optionally a calcium-binding compound
11 are combined. The calcium binding compound can be used to ensure that no calcium in the
12 sample affects the accuracy of the assay. The quantity of α -amylase activity formed due to
13 chloride ion and sodium ion (and calcium ion) in the bodily fluid sample is then assayed.
14 Finally, the quantity of sodium ions is determined by reference to the assay of α -amylase..
15 Whereas the chloride ion determination reagents and methods are designed so that chloride
16 ion concentration is the limiting factor in the enzymatic reactions, the sodium ion
17 determination reagents and methods are designed so that sodium ion is the limiting factor.
18 Sodium ion determination methods also include the additional step of accounting for any
19 calcium ion that may be present in the sample. If no calcium-binding compound is used, it
20 is preferable to determine the amount of calcium in the sample before performing the above
21 assay.

22 The following examples are given to illustrate the present invention, and are not
23 intended to limit the scope of the invention.

24

EXAMPLE 1

A calcium-containing substrate reagent and an enzyme reagent were prepared according to Table 1 and Table 2 respectively below. Assays were conducted with and without the addition of 30 mM sodium citrate to the enzyme reagent. The results are plotted in Figure 1 with the rate in A/min plotted versus chlorine concentration in mM. It was determined that the addition of sodium citrate in the enzyme reagent resulted in higher reaction rates than without the sodium citrate.

Table 1

SUBSTRATE REAGENT	CONCENTRATION
CNPG3	5 mM
TEA	80 mM
LiOH	100 mM
EDTA	30 mM
Ca Acetate	7 mM
Triton X	0.19 %
Trehalose	2 %
PEG 8000	4 %
BSA	2 %

Table 2

ENZYME REAGENT	CONCENTRATION
Amylase	15000 U/L
MES	10 mM
LiOH	30 mM
EDTA	10 mM
Dextran	3 %
Trehalose	5 %

EXAMPLE 2

A further study was conducted to determine whether the activation of α -amylase was due to the sodium ions or the citrate ions. Four assays were conducted, one with no sodium

ions or citrate ions in the enzyme reagent, one with sodium citrate in the enzyme reagent, one with sodium acetate in the enzyme reagent, and one with citric acid in the enzyme reagent. The substrate and enzyme reagents were otherwise prepared as indicated in Tables 1 and 2. Thus, sodium acetate served as the alternate source for sodium and citric acid served as the alternate source for citrate. As illustrated in Figure 2, the assay with sodium acetate showed a similar activation to the use of sodium acetate while the citric acid assay showed no activation.

EXAMPLE 3

Another study was conducted with varying concentrations of sodium acetate. Assays were conducted with sodium acetate concentrations of 0.0 mM, 22.5 mM, 45.0 mM, 90.0 mM, and 180.0 mM. The substrate and enzyme reagents were otherwise prepared as indicated in Tables 1 and 2. Five chloride concentrations were used, varying from approximately 68 mM to approximately 139 mM. As illustrated in Figure 3, higher concentrations of sodium acetate resulted in increased activation rates.

EXAMPLE 4

A sodium-containing substrate reagent and an enzyme reagent were prepared with the following components as listed in Table 3 and Table 4 respectively below. In this study, sodium citrate was added directly to the substrate reagent at concentrations of 30 mM and 60 mM. Five assays were run for each sodium citrate concentration at chloride concentrations of from about 68 to about 139 mM. The study was conducted to compare the substrate

1 reagent with different sodium citrate concentrations. As illustrated in Figure 4, the samples
2 with higher sodium citrate concentrations showed higher activation rates in each assay.

3 Table 3

SUBSTRATE REAGENT	CONCENTRATION
CNPG3	5 mM
TEA	50 mM
LiOH	40 mM
EDTA	40 mM
Sodium citrate	30 mM, 60 mM
Trehalose	2 %
PEG 8000	4 %
BSA	2 %

11 Table 4

ENZYME REAGENT	CONCENTRATION
Amylase	~15000U/L
MOPS	10 mM
LiOH	40 mM
EDTA	20 mM
Trehalose	2 %
PEG 8000	4 %
BSA	4 %

17 The present invention may be embodied in other specific forms without departing
18 from its spirit or essential characteristics. The described embodiments are to be considered
19 in all respects only as illustrative and not restrictive. The scope of the invention is,
20 therefore, indicated by the appended claims rather than by the foregoing description. All
21 changes which come within the meaning and range of equivalency of the claims are to be
22 embraced within their scope.

23 What is claimed and desired to be secured by United States Letters Patent is:

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